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Description

DNA-Sequences coding for a glucose-translocator, plasmids, bacteria, yeasts and plants containing this transporter.

The presented invention relates to DNA sequences from Zea mays (maize), Solanum tuberosum (potato) and Spinacia oleracea (spinach), which contain the coding region of a glucose-translocator whose introduction into a plant genome changes the development and allocation of carbon molecules in transgenic plants, as well as plasmids, yeasts, and bacteria containing these DNA sequences and transgenic plants in which, by introducing these DNA sequences, changes of the activity of the glucose translocators are caused which leads to changes of the carbon partitioning. Furthermore, the invention relates to the use of the described sequences of the glucose-translocator to identify related translocators from other plants (angiosperms, gymnosperms, and algae) through hybridization with low stringency or through PCR techniques, as well as to the use of the glucose-translocator as a target for herbicides.

Plant photosynthesis is the greatest synthesis process on the earth and the only process, which increases the total supply of useful energy on our planet. More than 10 billion tons of carbon is stored yearly in carbohydrates and other organicmaterials as energy and lastly as our sole source of food production. This amount of energy corresponds to about 5% of all known fossil energy sources. The photosynthesis forms therefore the basis of our existence. During this process, which takes place in photosynthetically active chloroplasts, atmospheric CO_2, inorganic phosphate and water with the help of the products of the light reactions, adenosine triphosphate (ATP) and reducing equivalents (NADPH), synthesize a carbon molecule having 3 carbon atoms, triose phosphate. This primary product of CO_2 fixation is transported from the chloroplast through a special translocator (triose phosphate /phosphate translocator, TPT; Flügge et al., 1989, EMBO J. 8: 39-46; Flügge et al., 1991, Nature 353: 364-367) and in the cytosol is converted to sucrose (cane sugar) by a succession of sequential

reactions and exported. During the biosynthesis of sucrose, the inorganic phosphate is released and transported back into the chloroplast by the above mentioned triose phosphate /phosphate translocator. Phosphate is regenerated into ATP by a reaction catalyzed by light-driven ATP synthesis. It is evident that the exchange of the daily fixed carbon with inorganic phosphate, mediated by the triose phosphate /phosphate translocator, connects the two compartments of the chloroplast and cytosol.

During the light period, photosynthetically formed triose phophate can also be transformed into starch, the so called transitory starch. This process resides in the chloroplast and serves for the synthesis of this transient carbon polymer, which is remobilized during the night period. Furthermore carbon can be assimilated by this process even if the biosynthesis of the sucrose in the cytosol is not as fast as the netto CO_2-assimilation. During the following dark period, starch polymers are degraded and the products of starch mobilization are exported from the chloroplast and synthesized into transport species of photoassimilates, mainly, sucrose, and exported from the leaf. In this manner, it is guaranteed that the non-green tissues are continually supplied both day and night with photoassimilates.

The mobilization of transient starch can be carried out by phosphorolytic as well as hydrolytic degradation. In the first case, hexose phosphates are produced and converted to triose phophates, which are then exported out of the chloroplast by the triose phosphate/phosphate translocator. In the cytosol, triose phophate is further metabolized to sucrose. These reactions include triose phophate isomerase and aldolase to make fructose-1,6-bisphosphate from glyceraldehyde3-phosphate and dihydroxyacetone-phosphate. These reversible reactions proceed in the direction of fructose-6-phosphate by loss of a phosphate group from fructose-1,6biphosphate. Based on its state of activation, fructose-1,6-biphosphatase (FBPase) can serve as a regulatory enzyme for sucrose biosynthesis. FBPase is under the strong control of fructose-2,6-biphospahte, an efficient negative effector. The concentration of this FBPase-inhibitor is under strong diurnal regulation, being low during the day and high in the dark. Such that, to a large extent, the activity of FBPase is eliminated in the dark

(Stitt 1990, Annu. Rev. Plant Physiol. Plant Mol. Biol. 41: 153-185). If the FBPase is not active, triose phophate exported from the plastids cannot be converted into sucrose. If this happens, the inorganic phosphate needed to export triose phophates is not produced. In plastids, inorganic phosphate is also necessary as a substrate for the phosphorolytic degradation of starch. For this reason, it is generally accepted that the night time degradation of starch occurs by hydrolytic degradation. This conclusion is corroborated by the observation that a mutant from Flaveria linearis, defective in the cytosolic FBPase, does not show an obvious phenotype. This mutant possesses a reduced carbon export from the leaves during the day, but increased carbon export rates during the night (Zrenner et al., 1996, Plant J. 9: 671-681). Also, experiments with a high starch mutant TC265 support this supposition (see below). In addition, NMR-Experiments using deuterium labeled glucose show that glucose is preferentially exported from the chloroplasts in the night, bypassing the triose phospate/phosphate translocator and the cytosolic FBPase (Schleucher et al., 1998, Plant Physiol. 118, 1439-1445). It must be here noted that this may not occur in all plants. In some plants, starch mobilization may occur entirely by phosphorolysis (Stitt, 1978, Biochim. Biophys. Acta 544: 200-214).

In contrast to phosphorolytic breakdown of starch, hydrolytic breakdown of starch by amylase, maltase, etc. produces glucose, the building block of starch. glucose is exported from the chloroplast by a special transporter, the glucose-translocator. After being phosphorylated to glucose-6-phosphate by hexokinase, glucose-6-phosphate is converted to fructose-6-phosphate by phosphoglucoisomerase. This reaction sequence bypasses the FBPase reaction. A further molecule of glucose-6-phosphate is converted to glucose-1-phophate by phosphoglucomutase and then to UDP-glucose by UDP-glucose pyrophosphorylase. UDP-glucose and fructose-6-phosphate are converted to sucrose phosphate by sucrose phosphate synthase and converted to sucrose by sucrose phosphate phosphatase.

During photosynthesis, newly formed photoassimilates move out through the TPT, but at night the glucose-translocator plays a decisive role in exporting mobilized photoassimilate. In the dark, the glucose-translocator connects metabolism between the

two compartments of the stroma and the cytosol. The essential role of the glucose translocator for mobilization of the products of starch degradation will be established through the following experiments.

Recently, it has been possible to make transgenic plants with a modulated activity of TPT. The inhibition of this transporter in planta by means of expression of anti-sense RNA leads to a lessening of the export of primary assimilates, 3phosphoglycerate and triose phophate, which under these circumstances remain in the chloroplast and are directed into the biosynthesis of starch, which accumulates in the chloroplasts (Riesmeier et al., 1993, PNAS 90: 61-64; Heineke et al., 1994, Planta 193: 174-180). It can be further shown that the accumulatedstarch can be mobilized in these plant species during the night (potato; Heineke et al., 1994 Planta, 193: 174-180), or already during the day (tobacco; Häusler et al., 1998, Planta 204: 366-376). As a result of this lowering of TPT activity in transgenic plants, starch mobilization follows the hydrolytic pathway and the export of free glucose occurs by the glucosetranslocator. It can also be shown that in these TPT plants, the activities of α-amylase, hexokinase, and glucose-translocator activity is three-times higher than in control plants.

The conclusion that the products of starch mobilization from the chloroplast appear mainly as glucose can also be established through the biochemical characterization of a starch excess Arabidopsis-mutant (TC26, defective in the sex1-gen), which shows a constant high starch content (Caspar et al., 1991, Plant Physiol. 95: 1181-1188; Trethewey and ap Rees, 1994, Biochem. J. 301: 449454). On the basis of its enzymatic make up, this mutant is able to degrade starch, but it is unable to export the products of starch degradation from the chloroplast. It can be shown that in the TC26 mutant the TPT is quite functional but the mutant is unable to transport glucose from the chloroplast. The alleged failure to transport glucose leads to an accumulation of the products of starch-degradation and thus to a re-synthesis of new starch in combination with the observed high starch level. Taken together, the discussed experimental evidence establish the decisive significance of the glucosetranslocator in the export of the mobilized carbon from the mobilized starch. The glucosetranslocator

was first described in 1977 (Schäfer et al., 1977, Plant Physiol. 60: 286-289), but a molecular and biochemical characterization of this translocator remains to be made.

The process of photoassimilate production occurs in source leaves and serves to provide the non-green parts of the plant, the sink organs, such as the roots, tubers and fruits and also the harvestable parts of the plant. In cultivated plants such as potatoes, sugar beet and cereals, the transport of carbohydrates proceeds from the source to the sink tissue through sieve tubes (phloem) principally in the form of sucrose. Sucose is transported out of the phloem into the cells of the sink tissue (phloem unloading). The transport either proceeds directly, that is, symplasticly, between the phloem and the sink tissue by way of plasmodesmata or apoplasticly, by which sucrose is effluxed into the cell wall and then taken up into the cells. Alternatively, sucrose in the apoplast can be broken down into hexoses by a cell wall specific invertase and then transported into the cells of sink tissue. Following uptake in the form of sucrose, the sugar is broken into hexoses (glucose and fructose) in the cytoplasm of the cells of the sink tissue. This can happen either by invertase or by sucrose synthase. All the breakdown products of sucrose are finally converted to hexose-phosphate (glucose-6-phosphate, glucose-1-phosphate).

In most plants, glucose-6-phosphate serves as substrate for starch synthesis, in which the whole reaction sequence is localized in the stroma of the amyloplast of the sink tissue. These organelles are enclosed by two lipid bilayer membranes. The outer membrane is unspecifically permeable to small molecules, because of the presence of pore forming proteins (porins). One such non-specific porin present in the outer amyloplast membrane was recently identified by us and its amino acid sequence of the protein determined (Fischer et al., 1994, J. Biol. Chem. 269: 25754-25760). The inner membrane is the permeability barrier between the cytosol and the plastid and is on the basis of its lipid bilayer impermeable to highly charged molecules, such as hexose phosphate. The principal way of traversing the inner membrane for high charged molecules is through the mediation of specific transport systems.

Glucose-6-phophate is imported in to the plastids by a transporter glucose-6 phosphate/phosphate translocator (GPT), which was recently described by us

(Kammerer et al., 1998, Plant Cell 10:105-117). Inside the plastid, the imported glucose-6-phosphate can then be used for the biosynthesis of starch. The import of glucose-6 phosphate into amyloplasts follows an exchange mechanism, i.e. in exchange of inorganic phosphate which is liberated during the course of the synthesis of starch. Glucose-6-phosphate is converted to glucose-1-phosphate which is then converted to ADP-glucose, the substrate for starch synthesis, by ADP-glucose-pyrophoshorylase (AGPase). Recent work has shown that in the endosperm of certain cereals (barley and maize), the AGPase is localized in the cytosol and the ADP-glucose produced by this reaction is then imported for starch synthesis (Denyer et al., 1996, Plant Physiol. 112: 779-785).

The exception to this may be the fat-accumulating plastids ofRicinus communis. In this case there exist hints that glucose may be taken up directly from the cytosol via a glucose-translocator to the plastids and is there phosphorylated by means of a plastidic hexokinase (Dennis and Miernyk, 1982, Annu. Rev. Plant Physiol. 33: 27-50) to give glucose-6-phosphate.

The imported glucose-6-phosphate is also a starting point for the oxidative pentose-phosphate pathway (OPPP), which provides reducing equivalents mainly for the on-going reduction of nitrate and nitrite to ammonia, which is used for the biosynthesis of amino acids and proteins. In this case, the transport of glucose-6 phosphate occurs in exchange with triose phophate, the products of the OPPP (Kammerer et al., 1998, Plant Cell 10:105-117).

Storage starch formed in amyloplasts and transitory starch formed in chloroplasts undergoes continuous synthesis and degradation (Stitt and Heldt, 1991, Biochim. Biophys. Acta 638: 1-11). If the synthesis of starch is greater than mobilization, then the starch will be laid down for long term storage for fixed carbon. Starch, which is laid down in the harvestable parts serves as the basis of animal/human nutrition (e.g. starch in potato tubers and corn kernels). During germination of seeds, starch is remobilized and the products of starch degradation serve as fuel for the emerging seedling. It is often seen that as in the above example of the export of mobilization of chloroplast

transitory starch, the glucose-translocator also plays a key role in the mobilization of storage starch of amyloplasts.

As can be seen from the above said transporters, such as the chloroplastidic glucose-translocator, which catalyzes the transport of fixed carbon from the chloroplast, the sucrose translocator, which loads the sieve tube with sucrose, the transport species of photoassimilate, the glucose-6-phosphate translocator of the amyloplasts, which provides transport substrates for starch synthesis, or the glucose-translocator, which allows the breakdown products of starch degradation to leave the chloroplast, these all have a decisive role in the allocation of photoassimilates from the source to the sink organs. A change in the activity of specific membrane transporters can have great consequences on the metabolic capacity of plants. Recently it was shown that the effectiveness of the photosynthetic carbon reduction (light reactions and Carbon Cycle) can be drastically influenced by the activity of the triose phosphate translocator (see above). Also, a reduction in sucrose transport activity as a result of an anti-sense inhibition verifies the role of sucrose transporters in phloem translocation (Riesmeier et al., 1992, EMBO J. 11: 4705-4713). This leads to an extreme phenotype in the plant; noticeably smaller, leaves are damaged, and the plants, in the case of potatoes, barely form tubers, that is, the supply of photoassimilates to the sink tissues is greatly reduced (Riesmeier et al., 1994, EMBO J. 13: 1-7).

For export, the glucose-translocator assumes a central role in the transport of the mobilization of the products of starch hydrolysis by photosynthetically active and non green plastids. Successful cloning of the translocator by means of the described sequence can alter the activity of these translocators in plants and opens up the possibility of increasing the starch content of plants and thereby dry weight.

As Stark et al. (Science 258: 287-292) have shown, the flux in the direction of starch synthesis in potato tubers can be raised by the overexpression of a ADP-glucose-phosphorylase (AGPase). For these experiments, an enzyme from E. coli was inserted, which was shown by a detailed analysis of the transgenic plants not to be metabolically controlled by the ratio of 3-phospho-glycerate/ phosphate. This insertion resulted in increased carbon flux into starch formation and also resulted in a higher

turnover of starch, because of increased mobilization of starch (Sweetlove et al., 1996, Biochem. J. 320: 493-498).

It therefore follows that, in order to attain a higher level of starch in plants, the turnover of starch must be lowered, i.e., the breakdown of starch and further metabolism and export of the breakdown product, glucose, must be inhibited. Plants with a reduced activity of the glucose-translocator can no longer export the products of amylolytic starch breakdown, glucose. Consequently, glucose from starch breakdown will be sealed in and, respectively, the further breakdown of starch product will be inhibited and the starch level increased. Such a process can be seen with the high starch mutant TC265, which has, due to a deficiency in the glucose-translocator, a higher level of starch.

An increase in the starch content of potato tubers will be significant for industries that use starch, as also for the nutritional industries, since potatoes with a higher starch content will contain a smaller amount of oil during the processing of potato chips, french fries etc and the caloric content of the end product will be lessened. A similar strategy can most probably be transferred to grains of other starch producing plants, such as, maize, wheat, and barley.

If we succeed in making potatoes with a reduced activity of glucose translocators, then we can also avoid the loss on long term storage, i.e. "Cold sweetening". Potatoes are stored at low temperatures (6-8 °C) to avoid germination. This leads nevertheless to the undesirable effect of the accumulation of soluble sugars, called "Cold-sweetening". The build-up of reducing sugars is the basis of the destructive Maillard reaction, a wasteful, negative by-product of the further processing of potatoes in the manufacturing of chips, etc. The formation of soluble sugars, the hydrolytic products of starch, which accumulate outside the chloroplast, can be hindered by manipulating the glucose translocator. This translocator which is connected with the export of the products of starch mobilization can be sealed off in potatoes during the storage phase. These effects can be amplified through a simultaneous reduction of the recently described glucose-6-phosphate/ phosphate translocator, which exports out of the plastid the phosphorolytic breakdown products of starch breakdown which are C3

compounds which are phosphorylated on one end (Kammerer et al., 1998, Plant Cell 10: 105-117).

As mentioned above, the export of the molecules of photosynthesis formed during the day occurs predominantly through the TPT. Experiments with transgenic plants in which the TPT has been repressed by expression of anti-sense RNA or increased shows that the TPT can limit photosynthesis and the biosynthesis of starch, particularly under high light and optimal CO_2 (Häusler et al., 1998, Planta 204: 366 376). It is conceivable that an overexpression of the glucose-translocator can bring about an increased efflux of fixed carbon from the chloroplast. This is especially true if there is simultaneous overexpression of ADP glucose phosphorylase which leads to increased starch turnover by a higher starch breakdown (Sweetlove et al., 1996, Biochem. J. 320: 493-498). The fixed carbon will have the alternative of exiting the chloroplast by the glucose-translocator.

The targeting of the plastidic translocator into the inner envelope of the plastid requires an expressed presequence ("targeting sequence"), which correctly directs the attached mature protein to the plastid (Keegstra et al., 1989, Annu. Rev. Plant Physiol. Mol. Biol. 40: 471-501; Lubben et al., 1988, Photosynthesis Res. 17: 173-194; Flügge, 1990, J. Cell Sci. 96: 351-354). In addition, to the needed leader sequence for "plastid targeting" there is in the mature part (the part of the protein that remains after the excision of the leader sequence by a specific protease) of the envelope protein still further information, which is responsible for the specific insertion of the protein in the plastid membrane and prevention of the transport of the envelope protein through the envelope into the plastid stroma, or in the case of the chloroplast, the thylakoid membrane (Knight and Gray, 1995, Plant Cell 7: 1421-1432; Brink et al., 1995, J. Biol. Chem. 270: 20808-20815). Other work shows further, for example, in the case of a mitochondrial carrier, the ADP/ATP translocator, that this protein could not be directed to the plastid or was only with a very poor efficiency inserted into the plastid inner membrane. The insertion of even a hybrid protein, consisting of a plastidic presequence (containing the information for a plastidic address) and this mitochondrial carrier, showed only a slightly higher increase in incorporation into the plastid inner membrane

over the authentic protein (Silva-Filho et al., 1997, J. Biol. Chem. 272: 15264-15269; unpublished observations). Since for correct insertion the protein/lipid ratio is important and the plastidic inner membrane has a unique lipid matrix than other cell organelles and also bacteria (Joyard et al., 1991, Eur. J. Biochem. 199: 489-509), it is very uncertain that a transporter from another system having similar function can be incorporated into the plastid inner membrane in a suitable conformation and orientation.

Therefore, given the present state of the technique, it is not possible with the help of known plastid targeting sequences to integrate functional mitochondrial or procaryotic transporters into the inner plastid membrane. Given the present state of the technique, for the construction of above mentioned plants, it will be necessary to clone and to use the authentic plastid glucose-translocator DNA sequence.

Although the transport system catalyzes the transport of glucose, its primary structure is probably considerably different from the glucose-translocators of the plasma membrane. These transporters belong to a large gene family of monosaccharide translocators (Sauer and Tanner, 1993, Bot. Acta 106: 277-286; Weig et al., 1994, J. Plant Physiol. 143: 178-183).

As mentioned above, physiological and biochemical studies of the high-starch Arabidopsis mutant TC265 indicated that this mutant is defective in the chloroplastidic glucose-translocator. Experiments to identify the corresponding gene (sex1) via a map based cloning approach showed that this gene can be localized to chromosom 1 (crossing of the mutant (ecotype Columbia) with the wildtype ecotype Landsberg erecta; unpublished own results). This genomic region, however, does not contain a sequence coding for a putative glucose-translocator.

In order to determine the primary structure of the plastidic glucose-translocator, one must characterize, purify and isolate the transport protein through a number of difficult biochemical procedures. We succeeded in obtaining from the chloroplast envelope of spinach this protein by means of chromatographic methods and by combining substrate protection along with labelling with a radioactive inhibitor N-ethylmaleimide. The protein was identified as a component of the inner chloroplast envelope with an apparent molecular mass of 43.000 Dalton.

The N-terminus of the translocator was chemically modified and not accessible to N-terminal protein sequencing by Edman degradation. It is notable that the protein could be isolated adequately through preparative SDS-Polyacrylamideelektrophoresis and cleaved with protease (Lys-C) in order to obtain internal peptide sequences. (see Example 1). In this way it was possible to obtain the following peptide sequence: (1) KGRSLEEIELASPAV. It was by means of 3'-RACE-method (rapid amplification of cDNA ends) and utilization of a degenerate (modeled from the sequenced peptide) synthetic oligonucleotides, that a specific PCR-fragment was obtained. This fragment was used to screen a cDNA library from spinach leaves and finally to isolate a complete cDNA clone (1864 base pairs) coding for the precursor protein of the chloroplastic glucose-translocator with a molecular mass of 57.6 kDa (see Example 2). This cDNA from spinach was subsequently used to isolate corresponding cDNA clones fromArabidopsis thaliana, Zea mays (maize), Solanum tuberosum (potato) and Nicotiana tabacum (tobacco). Experiments to complement the TC265 mutant phenotype using the cDNA sequence from spinach failed (unpublished, own observations) indicating that the glucose-translocator-gen cannot be isolated by map-based cloning of thesex1-gen. We could further show that the glucose-translocator-gen from Arabidopsis thaliana is localized to chromosome 5. A second, homologous gene could be localized to chromosome 1, about 4 cM downstream of the sex1-locus (own, unpublished observations).

A comparison of an entire DNA-sequence with the sequences from the data bank showed only low similarities with known glucose-translocators of the plasma membrane (<30%) and with bacterial hexose transporters (about 45%). Further it showed about 73% homology with a hypothetical sugar transporter ofPrunus armeniaca (apricot). The derived molecular mass of this transporter was about 49.8 kDa. Here was obviously the basis for an error, namely that this protein also has an N-terminal sequence, which could serve as an insertion signal for the plastid. The assignment of the identified gene in apricot to a cell membrane or an organelle could not be established. We can show, however, that the chloroplast glucose-translocator is synthesized as a precursor protein

and posttranslationally the N-terminal sequence is split off following import into the chloroplast and insertion into the inner envelope membrane (see Example 3.

The presented invention provides DNA sequences from plant genomes that code for plastidic glucose-translocators; the information contained in the nucleotide sequences, with introduction and expression in plant cells, produce an mRNA and by means of this mRNA can produce a glucose-translocator activity in cells or suppress a endogenous glucose-translocator activity. The invention relates in particular to the DNA sequences from Zea mays (maize), Solanum tuberosum (potato) and Spinacia oleracea (spinach) with nucleotide sequences shown in the sequencing protocols (see below).

The invention relates furthermore to DNA sequences, which with the DNA sequences (sequencing protocols) or parts thereof, or derivatives, which were derived through insertion, deletion or substitution of these sequences, hybridize and code for a plastidic protein, which possesses biological activity of a glucosetranslocator.

Furthermore, the invention relates to the use of the DNA sequences of the invention or parts thereof or derivatives, which were derived through insertion, deletion or substitution of these sequences, for transformation of procaryotic and eucaryotic cells. In order to express the glucose-translocator in transformed cells, the DNA sequences of the invention can be combined with vectors and thereby with control elements for the expression in procaryotic and eucaryotic cells (seeExample 3 and 5). Such control elements are, on the one hand, transcription promoters and on the other hand transcription terminators. With these vectors, embryonic cells can be transformed with the goal of expression of an messenger ribonucleic acid (mRNA) which allows the synthesis of a plastidic glucose-translocator in the transformed cells or with the goal of not expressing using inversely oriented (anti-sense) mRNA, which inhibits the synthesis of the endogenous glucose-translocators. For this goal short fragments of the DNA sequences of the invention with a relatively high degree of homology (greater than 65% homology) can be used. Repression can also achieved by overexpression of homologous DNAs (cosuppression). Also expression of the endogenous glucose translocator can be inhibited through the expression of ribozymes constructed for this purpose under the direction of the DNA sequences of the invention, by using insertion

mutagenesis, or as soon as the described technology exists, by means of homologous recombination ("knockout" mutant). By means of tissue-specific controlled expression of mRNA expressing the sequence of the present invention of a plant glucose-translocator, it is possible to change the plant's carbohydrate metabolism; therein lies the economical significance, that an improved efflux of photoassimilates from the chloroplasts can be attained or the breakdown products of starch mobilization in the plastid can be retained.

Plants can thus be created that contain an elevated starch content and/or, as in the case of potatoes, show a reduced storage loss. These modifications probably improve the nutritional value of the plant and are of much economic interest. The actual effects could be increased by combining the tissue-specific expression of the glucose-translocator in leaves or in the heterotrophic sink tissues with that of other enzymes/translocators of carbon/nitrogen metabolism. (Simultaneous expression of different genes to increase starch content or to lessen storage loss in potatoes).

Furthermore, the heterologous expression of the described sequence in yeast cells (see Example 4 and Loddenkötter et al., 1993, Proc. Natl. Acad. Sci. USA 90: 2155-2159) allows structure-function studies of the glucose-translocator, which could lead finally to the development of a specific inhibitor for this protein; in particular as regards herbicide development; inhibition of this protein which has a key function in essential metabolism will be lethal for plants. In addition, these structure-function studies form the basis for site-directed mutagenesis of the substrate binding site of the translocator, in order to alter its substrate specificity.

The procedure for genetic modification of dicotyledonous and monocotyledonous plants is already known (Gasser and Fraley, 1989, Science 244: 1293-1299, Potrykus, 1991, Annu. Rev. Plant Physiol. Plant Mol. Biol. 42: 205-225). In order to express coded sequences in plants, these must be linked with transcriptional regulatory elements. Such elements, named promoters, are known (e.g., Koster-Toepfer et al., 1989, Mol. Gen. Genet. 219: 390-396). Furthermore the coding regions must be provided with a transcription termination signal, so that it can be correctly transcribed. Such elements have also been described (Gielen et al., 1989, EMBO J. 8: 23-29). The transcriptional starting region can be endogenous, that is homologous, as well as foreign, that is

heterologous for the host plant. The termination sequences are interchangeable with each other. The DNA-sequence of the start and termination sequences can be put together synthetically or naturally occurring or can contain a mixture of synthetical or naturally occuring DNA parts. For the preparation of the introduction of the foreign gene in higher plants, a large number of cloning vectors are available, which contain a replication signal for E. coli and a maker, which permits selection of transformed cells. Examples of vectors are pBR322, pUC series, M13 mp-series, pACYC 184, and so forth. Depending on the introduction method of the desired gene in plants, additional DNA sequencing may be necessary. In order to use the Ti or the Ri-plasmid for the transformation of plants, at least the right region and more often the right and the left regions of the Ti and Ri-plasmid T-DNA must be attached to the genes to be introduced. The use of T-DNA for the transformation of plant cells has been intensively studied and described (Hoekema, 1985, In: The Binary Plant Vector System, B.-V. Kanters, Ablasserdam, Chapter 5; Fraley et al. Critical Rev. Plant Sci. 4: 1-46; An et al. 1985 EMBO J. 4: 277-287). If the introduced DNA is integrated into the genome, it is stable as a rule and remains in the descendants of the transformed cell. It normally contains a selection marker, which confers on the transformed plant cells resistance to biocides (such as Basta) or an antibiotic (such as kanamycin, bleomycin, or hygromycin). The individual introduced marker will allow accordingly the selection of the transformed cells from the other cells, which do not contain the introduced DNA.

For the introduction of DNA into plants host cell, a number of techniques are available, besides the Agrobacterium-mediated DNA transfer. These techniques include the transformation of protoplasts, microinjection of DNA, electroporation, as well as ballistic methods. From the transformed plant material, whole plants can be regenerated from a suitable selection medium. The thus derived plants can then be tested with current molecular biological methods for presence of theIntroduced DNA. These plants can be grown normally and can be crossed with plants which possess the same or different genetic backgrounds. Thence, the resulting hybrid individuals have the corresponding phenotypical properties.

The DNA-sequences of the invention (or derivatives or parts of these sequences) can be incorporated into plasmids, which permit a mutation or a sequence variation through recombination of the DNA sequences in procaryotic or eucaryotic systems. Thereby the specificity of the glucose-translocator can be altered, for example, in the direction of a change in the affinity for fructose or galactose.

Furthermore an insensitivity of the glucose-translocator for a specific herbicide could be attained. With the help of standard methods (Sambrœk et al., 1989, Molecular cloning; A laboratory manual, 2. edition, Cold Spring Harbor Laboratory Press, NY), exchange and/or deletion of a single base in the DNA molecule can be undertaken and/or synthetic or natural sequences added. Adapter or linkers can be used for combining DNA fragments with each other. Further manipulations can be inserted which create suitable restriction sites or remove unwanted DNA regions. In case insertions, deletions or substitutions, for example, transitions or transversions are conceivable, in vitro mutagenesis, primer repair, restriction or ligation can be employed. Analytical methods that can be employed comprise DNA-sequencing, restriction sites analysis and other biochemical/molecular methods, for example, the expression of modified proteins in yeast and the study of the modified transport characteristics in artificial liposomes (see Method 4; Loddenkötter et al., 1993, Proc. Natl. Acad. Sci. USA 90: 2155-2159; Fischer et al., 1997, Plant Cell 9: 453-462; Kammerer et al., 1998, Plant Cell 9: 453-462) or the study of modified transport characteristics using tissue homogenates from transgenic plants (Flügge and Weber, 1994, Planta 194: 181-185).

The DNA-sequences of the invention(or parts or derivatives of these sequences) can be used to isolate, according to standard methods (especially hybridization of a cDNA library at low stringency with the identified DNA sequences or parts of the DNA sequences as a probe or the preparation of probes for stringent or low stringency screening approaches through derivation of degenerate and/or non degenerate primers of the identified DNA-sequences for PCR experiments with DNA or cDNA from other plants), from the genomes of other plants similar sequences, which code for glucose transporting proteins.

The DNA sequences of the invention code for protein domains, which target the cytoplasmatically made proteins specifically to the plastids and prevent insertion into other membrane systems in the cell. This protein domain, which directs the proteins coded by the DNA sequences of the invention to palstids, lies within the first 90 amino acids of the precursor protein, is not necessary for the transport function of the protein and is removed following the successful insertion of the protein into the inner membrane. Through exchange of this "plastid targeting" sequence with a known "targeting" sequence, for example, for mitochondria, the translocator protein could be directed to another membrane system of eucaryotic cells and can there possibly change the transport character of the respective membrane. In addition the "plastid-targeting" sequence of the glucose-translocator or endogenous domains of the mature protein can be used to direct foreign proteins (e.g. bacterial transport proteins or transporters from yeast) to plastids or to the plastidic inner membrane of plant cells.

To obtain a better understanding of these teachings of the present invention, the most important methods used according to the present invention are explained in more detail below:

1. Cloning method

For cloning, the phage LambdaZAP II as well as the phagemid pBluescript II SK (pBSC) (Short et al., 1988, Nucl. Acids Res. 16: 7583-7600) were employed.

For the transformation of yeast, the vector pEVP11 (Russel and Nurse, 1986, Cell 45: 145-153) was employed.

For plant transformation, the gene construction was cloned into the binary vector pBinAR (Höfgen and Wilmitzer,1990, Plant Sci. 66: 221-230).

2. Bacteria and yeast stocks

For the pBluescript SK (pBSC) phagemid as well as the pEVP11 and the pBinAR constructs, the E. coli strain DH5α (Hanahan et al., 1983, J. Mol. Biol. 166: 557-580) was employed.

The transformation of pBinAR constructs into tobacco plants was carried out with the help of Agrobacterium tumefaciens strain C58C1, pGV2260 (Bevan, 1984, Nucl. Acids Res. 12: 8711-8720).

3. Transformation of Agrobacterium tumefaciens.

The transfer of DNA into Agrobacteria was achieved through direct transformation according to the method of Hoefgen and Wilmitzer (1988, Nucl. Acids Res. 16: 9877). The plasmid DNA of transformed Agrobacteria was isolated according to the method of Birnboim and Doly (1979, Nucl. Acids Res. 7: 1513-1523) and after appropriate restriction cleavage analyzed electrophoretically for exactness and orientation.

4. Plant transformation

For transformation, 15 small leaves from a sterile tobacco culture were excised with a scalpel in pieces of about 1 cm in length. The leaf pieces were transferred into MS medium containing 2% glucose and inoculated with a transformed Agrobacterium tumefaciens culture of bacterial cells. After 30 minutes of incubation, the leaves were incubated with MS medium containing 1.6% glucose for two days in the dark at room temperature. Finally, the leaf pieces were transferred to a MS medium containing 2% sucrose, 2 mg/L kinetin, 500 mg/L Betabactyl®, 15 mg/L hygromycin (or 100 mg/L kanamycin) and 0.8% Bacto-Agar. After one week of incubation at 25 °C and 3000 lux illumination intensity, the concentration of Betabactyl® in the medium was reduced to half. Out of the leaf pieces, without the appearance of callus, grew small shoots, which developed roots upon transfer to a MS medium containing 2% sucrose (Suc), 250 mg/L Betabactyl® and 100 mg/L kanamycin. After root development, the shoots were transferred to earth culture.

The transformation of Arabidopsis followed the method of Bechtold et al., 1993, C.R. Acad. Sci. 316: 1194-1199.

Depositions:

On 10 June 1998 and June 10 1999, the following phagemids contained in E. coli strains were deposited at the German Collection of Microorganisms (DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany):

Phagemid	pBSC-E43/30-3	(DSM 12243)
Phagemid	pBSC-Zm-pGT	(DSM 12862)
Phagemid	pBSC-St-pGT	(DSM 12863)

Example 1

Isolation of the glucose-translocator, production of peptide fragments of this translocator and construction of probes for screening cDNA libraries

Identification of the glucose-translocator on the intermembrane of spinach chloroplast took place through a combination of radioactive tagging experiments and substrate protection experiments. It can be shown that the transport of glucose into isolated chloroplasts can be inhibited by very low concentrations of the sulfhydryl reagents pCMBS and NEM.

Isolated chloroplasts were incubated in the presence or absence of glucose, maltose, or sorbitol with the radioactively tagged sulfhydryl reagent N-ethylmaleimide (NEM). Subsequently, chloroplast envelope membranes were isolated, separated by SDS-PAGE and then fixed, stained and the dried gel subjected to fluorography. It can be shown that a protein with an apparent molecular mass of 43 kDa on SDS-PAGE was differentially labeled under contrasting conditions.

Treatment of chloroplast envelope membranes with a mixture of chloroform and methanol (2:1) extracted the identified glucose-translocator together with four other proteins. The thick lipid containing extract was not directly accessible to separation on preparative SDS-PAGE, but had to be dilipidated first of all with hexane.

The separation of proteins on preparative SDS-PAGE was by Laemmli (1970, Nature 227: 680-685). Following detection of the protein by staining with Coomassie Brilliant Blue R-250, the 43 kDa protein band was cut out of the gel and cleaved with the endoprotease Lys-C (Fresco, 1979, Anal. Biochem. 97: 382-386). The resulting peptides were eluted from the gel and separated by HPLC (Eckerskorn and Lottspeich, 1989, Chromatographia 28: 92-94). The amino acid sequence of the purified peptide fractions was determined through automated Edman degradation in the gas phase (Eckerskorn et al., 1988, Eur. J. Biochem.176: 509-510). From the amino acid sequence of one of two peptides (peptide 1), two degenerate oligonucleotide sequences, coding for these amino acid sequences, were deduced and the corresponding DNA-fragment prepared through in vitro DNA synthesis.

Example 2

Cloning of the glucose-translocator

Spinach leaves were harvested in the middle of the light period and in the middle of the dark period and stored until RNA isolation at-80 °C. From these was polyA$^+$-RNA isolated and proceeding from this a cDNA library was established in vector LambdaZAPII.

In addition, the isolated RNA was reversely transcribed using a reverse transcriptase-polymerase-linked reaction (PCR) (RACE, Schaefer, 1995, Anal. Biochem. 227, 255-273). The first specific oligonucleotide was used as the primer for the first RACE reaction in combination with a "anchor(dT)15" primer. The obtained products wereused in a second PCR with the corresponding "nested" primers. From this, a specific PCR fragment (300 bp) was obtained.

About 300,000 clones of the cDNA library were screened using this PCR fragment (see example 1). Positive clones were purified according to standard methods and after preparation of the amplified phage DNA from the purified plaques, aEcoRI restriction digest resulted in the isolation of the insert, coding for the glucose translocator, which was verified though Southern blot analysis using the forementioned PCR-fragment as a probe. After in vivo excision of the phagemids from the phages with help of the filamentous helper phage ExAssist, the clones were analyzed through determination of the DNA sequence (Dideoxymethod: Sanger et al., 1977, Proc. Nat. Acad. Sci. USA 74: 5463-5467) and from these DNA sequences the primary structure of the glucose-translocator (GlcT) was deduced (clone pBSC-E43-30/3). The sequence of the peptides can be found in the total sequence of the translocator. The cDNA from spinach was subsequently used to isolate corresponding cDNA clones fromArabidopsis thaliana, Zea mays (maize), Solanum tuberosum (potato) and Nicotiana tabacum (tobacco) through screening of cDNA libraries at low stringencies.

Example 3

Targeting of the glucose-translocator precursor protein to chloroplasts and energy-dependent insertion of the mature protein into the inner envelope membrane

The in vitro transcription of plasmid pBSC-E43-30/3 was directed using the T7 RNA polymerase following the directions of the manufacturer (Pharmacia). Thein vitro translation followed in reticulocyte lysate (Boehringer-Mannheim) and the post ribosomal supernatant was used for protein transport experiments into intact spinach chloroplasts. The experiment was carried out in the dark or in the light; the assay contained import buffer (Flügge et al., 1989, EMBO J. 8: 39-46) and intact spinach chloroplasts (about 200 µg of Chlorophyll). After 30 min at 25 °C, the chloroplasts were washed and the envelope membranes isolated (Flügge et al., 1989, EMBO J. 8: 39-46), which were then analyzed by SDS-PAGE (Laemmli, 1970, Nature 227: 680685) and fluorography (Bonner and Laskey, 1974, Eur. J. Biochem. 46: 8488). It can be shown that the presequence of the glucose-translocator directed correctly the attached mature

protein to its target membrane, the inner chloroplast envelope; it was cleaved during the import process by a specific protease and the mature protein resulted. In the dark, the insertion of the translocator can be energized by the addition of ATP, however, in the absence of ATP no import is observed. In the light, the energy for the import of the protein can be provided by ATP generated by the photosynthetic phosphorylation; the import was independent from externally added ATP. While photosynthetic phosphorylation and the combined production of ATP was hindered through the addition of the uncoupler CCCP, protein import was also blocked. It could be shown further that the mature protein, when inserted into the inner envelope, was resistant to proteases: addition of proteases (e.g. thermolysin), which cannot permeate the outer envelope, had not effect on the mature protein when inserted into the inner membrane. A pre treatment of the chloroplasts with a protein (e.g. thermolysin) leads to a complete loss of binding and of the import of the translocator, which shows that the presequence ("targeting" sequence) of the translocator in the first place must be bound to a specific receptor on the outer membrane. Only after binding can the protein be inserted.

Example 4

Expression of the glucose-translocator from spinach in Schizosaccharoyces pombe

For the expression of the glucose-translocator in yeast cells, first the corresponding fragment was prepared by PCR using an oligonucleotide located at the cutting point of the presequence and the mature protein. This DNA fragment was cloned into the yeast expression vector pEVP11 and, after amplification of the construct in E. coli, transformed into leucine synthesis deficient S. pombe cells (Fischer et al., 1997, Plant Cell 9: 453-462; Kammerer et al., 1998, Plant Cell 10: 105-117), which were made competent through LiCl/PEG treatment (Ito et al., 1983, J. Bact. 153: 163168). Transformants were selected through selection on minimal medium without leucine, since the pEVP11-E43-30/3 construct grants the ability of yeast cells to grow on leucine-free medium.

Example 5

Transformation of plants with a construct for overexpression of the coding region of the glucose-translocator

From the phagmid pBluescript-E43-30/3 (pBSC-E43-30/3), containing the cDNA for the glucose-translocator isolated from spinach (see Method 2), the insert was removed through restriction digestion and cloned into the vector pBinAR (Höfgen and Wilmitzer, 1990, Plant Sci. 66: 221-230). After amplification of the resulting construct pBinAR-E43-30/3 in E. coli, the construct was transformed into Agrobacteria and these cells were then used to infect leaf segments of tobacco and potato.

The resulting transformants were analyzed by Southern blot analysis for the presence of the intact, not rearranged chimeric gene. Glc transport activity in the transformants was compared to that of control transformants (transformed with the vector pBinAR without insert) as well as the C/N ratio, photosynthesis, starch content and growth.